MYCOLOGIA
2023, VOL. 115, NO. 2, 187–205
https://doi.org/10.1080/00275514.2022.2157665

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Mitochondrial genomes in the iconic reindeer lichens: Architecture, variation, and synteny across multiple evolutionary scales

Jordan R. Hoffman [a,b], Kenneth G. Karol[b], Yoshihito Ohmura[c], Cloe S. Pogoda[d], Kyle G. Keepers[d], Richard T. McMullin[e], and James C. Lendemer[b]

[a]Department of Biology, The City University of New York Graduate Center, 365 5th Avenue, New York, New York 10016; [b]Institute of Systemic Botany, The New York Botanical Garden, Bronx, New York 10458-5126; [c]Department of Botany, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba 305-0005, Japan; [d]Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, Colorado 80309; [e]Research and Collections, Canadian Museum of Nature, PO Box 3443, Station D, Ottawa, Ontario K1P 6P4, Canada

ABSTRACT

Variation in mitochondrial genome composition across intraspecific, interspecific, and higher taxonomic scales has been little studied in lichen obligate symbioses. *Cladonia* is one of the most diverse and ecologically important lichen genera, with over 500 species representing an array of unique morphologies and chemical profiles. Here, we assess mitochondrial genome diversity and variation in this flagship genus, with focused sampling of two clades of the "true" reindeer lichens, *Cladonia* subgenus *Cladina*, and additional genomes from nine outgroup taxa. We describe composition and architecture at the gene and the genome scale, examining patterns in organellar genome size in larger taxonomic groups in Ascomycota. Mitochondrial genomes of *Cladonia, Pilophorus*, and *Stereocaulon* were consistently larger than those of *Lepraria* and contained more introns, suggesting a selective pressure in asexual morphology in *Lepraria* driving it toward genomic simplification. Collectively, lichen mitochondrial genomes were larger than most other fungal life strategies, reaffirming the notion that coevolutionary streamlining does not correlate to genome size reductions. Genomes from *Cladonia ravenelii* and *Stereocaulon pileatum* exhibited ATP9 duplication, bearing paralogs that may still be functional. Homing endonuclease genes (HEGs), though scarce in *Lepraria*, were diverse and abundant in *Cladonia*, exhibiting variable evolutionary histories that were sometimes independent of the mitochondrial evolutionary history. Intraspecific HEG diversity was also high, with *C. rangiferina* especially bearing a range of HEGs with one unique to the species. This study reveals a rich history of events that have transformed mitochondrial genomes of *Cladonia* and related genera, allowing future study alongside a wealth of assembled genomes.

ARTICLE HISTORY
Received 4 April 2022
Accepted 2 December 2022

KEYWORDS
Ascomycetes; Cladoniaceae; fungi; North America; organelle; transposable elements

INTRODUCTION

Lichens are a speciose, evolutionarily diverse collection of fungi unified by their shared lifestyle, a symbiosis between a fungus (mycobiont) and one or more photosynthetic microbes (photobionts; Lutzoni et al. 2001). Among the 995 lichen genera spread across 117 fungal families, *Cladonia* is among the most diverse, with over 500 species (Lücking et al. 2017). *Cladonia* is nearly cosmopolitan, with species occurring in a wide range of habitats, forming important components of many ecosystems as a critical food source for many animals, including large mammals such as caribou (Asplund 2010; Nugent 1990; Storeheier et al. 2002), stabilizing soils (Eldridge and Kinnell 1997; Eldridge et al. 2000; Rai 2012), and providing habitat for a vast microcosm (Noh et al. 2020; Siddiqi and Hawksworth 1982; Zawierucha et al. 2017). As a highly diverse genus of conspicuous macrolichens with immense morphological and chemical variation, *Cladonia* have been intensively studied from ecological, pharmacological, phylogenetic, and taxonomic perspectives (Lendemer and Hodkinson 2009; Pino‐Bodas et al. 2013; Stenroos et al. 2002; Stenroos et al. 2019). Yet, despite its overrepresentation in what is currently a budding lichen genomic research scene (Hoffman and Lendemer 2018), there have been to date remarkably few genomic studies of this genus (Armaleo et al. 2019; Junttila 2016; Park et al. 2013, 2014).

Phylogenetic and species delimitation studies in lichens, including those focused on *Cladonia*, have primarily utilized nuclear loci, with only two mitochondrial genes (cytochrome oxidase subunit 1 and the mitochondrial small-subunit ribosomal rRNA) widely employed

CONTACT Jordan R. Hoffman jhoffman2@gradcenter.cuny.edu
Supplemental data for this article can be accessed online at https://doi.org/10.1080/00275514.2022.2157665.

Published online 03 Feb 2023

(Hoffman and Lendemer 2018). Certain qualities of mitochondrial genes, such as conservation tied to critical biological function and atypical modes of inheritance (predominantly uniparentally in ascomycetes; Xu and Li 2015), make them informative in phylogenetics, population genetics, and phylogeography (Avise et al. 1987; Ballard and Whitlock 2004; Patwardhan et al. 2014). Recent research also demonstrated that mitochondrial genome content and architecture is highly variable in lichens, likely reflecting both complex evolutionary histories and coevolution with diverse symbionts (Pogoda et al. 2018). This work also highlighted an unusual abundance of introns and homing endonuclease genes (HEGs), a type of highly mobile genetic element, in lichen fungal mitochondrial genes (Pogoda et al. 2018, 2019).

One study has identified significant variation in genome architecture, introns, and HEGs within *Cladonia* (Birgham et al. 2018). However, less than 3% of *Cladonia* species were included, and intraspecific variation was not investigated. Thus, knowledge of mitochondrial genomic diversity, architecture, and evolution is limited, and mitochondrial genomes remain, on an intraspecific level, unstudied. Here, we provide the first detailed, interspecific and intraspecific comparative study of mitochondrial genomes, using the evolutionary framework of *Cladonia* and its relatives.

MATERIALS AND METHODS

Sample collection and identification.—Between 2016 and 2019, 73 specimens of 22 *Cladonia* species were collected from locations across North America and Japan (TABLE 1). Of these samples, 30 *C. rangiferina* were collected in replicate at 18 sites and 14 *C. submitis* were collected at four sites (SUPPLEMENTARY TABLE 1). Nine additional collections of *Pilophorus fibula* (Cladoniaceae), *Lepraria caesiella, L. eburnea, L. finkii, L. normandinoides, L. oxybapha, L. vaouoxii, Stereocaulon dactylophyllum*, and *S. pileatum* (Stereocaulaceae) were also sampled as outgroups to *Cladonia* based on the strongly supported sister relationship between families recovered by Miadlikowska et al. (2006). Those outgroup taxa, and an additional 31 species from eight genera, were sampled as part of the National Science Foundation Division of Environmental Biology (NSF DEB) awards 1145511 and 1542629, large-scale research projects examining lichen biodiversity gradients in the southern Appalachian Mountains of eastern North America (see Tripp et al. 2019). Collections were identified using standard morphological assessments with a dissecting microscope, chemical spot tests (K, C, KC, and P, as well as long-wavelength ultraviolet [UV] light; Brodo et al. 2001), and thin-layer chromatography (TLC) to determine lichen secondary metabolites present in the thalli and as confirmation of spot test results. TLC was performed using acetone extracts from thallus fragments, utilizing solvent C (Culberson and Kristinsson 1970) as modified by Lendemer (2011) and with a toluene:glacial acetic acid ratio of 200:30.

DNA extraction and sequencing.—For each sample, a 2-mL tube was filled with lichen thallus material. For species that produce podetia, a single podetium was used, unless one podetium was too small to reliably extract enough DNA for sequencing. For squamulose species, squamules were scraped into the vial until full. Squamules were only sampled from contiguous thalli identified to species to minimize the potential of unintentionally sampling multiple species, in the event that a nontarget species was present in the collection. Each sample was crushed while dry with a Qiagen TissueLyser machine (Hilden, Germany; Illumina, San Diego, CA), and DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle 1991; using pure chloroform in place of a 24:1 chloroform: isoamyl alcohol solution). Following extraction and Qubit DNA quantification and standardization, samples were submitted to Cold Spring Harbor Laboratories and the University of Wisconsin Biotechnology Center for library preparation and sequencing. This 150-bp paired-end sequencing was conducted on Illumina Next-Seq and Nova-Seq machines (San Diego, CA), producing metagenomes that averaged 40 million reads per sample.

Filtering, assembly, and annotation.—Raw reads were uploaded into Geneious Prime (2021) and paired. Paired reads were trimmed of remaining adapters and low-quality regions using the trim function with default settings. Trimmed reads were assembled de novo using the Geneious assembler under low-sensitivity settings. The top 300 consensus sequences were screened for fungal mitochondrial genes with the annotation function, using six complete *Cladonia* mitochondrial genome sequences as reference (SUPPLEMENTARY TABLE 1). Consensus sequences that returned similar regions to the reference mitochondria received iterative assembly, where the consensus was built at the ends continuously until the sequence began to repeat (a sign of circularization) or until no additional progress was made. This process continued until the genome was complete, upon which it was circularized and

Table 1. *Cladonia, Pilophorus, Lepraria*, and *Stereocaulon* samples used in this study, including isolate ID, collection location, assembled mitochondrial genome size, coverage, and Genbank accession number.

Isolate	Genus	Species	Collector	Collection number	Location	Country	Genome size (bp)	Coverage	Accession number
FEN-380	*Cladonia*	*apodocarpa*	J. Lendemer	48789	North Carolina	USA	50 382	251×	OL989721
SUBJ-972	*Cladonia*	*arbuscula*	J. Hoffman & Y. Ohmura	972	Honshu	Japan	57 826	2852×	OL989765
WRM1-ARB-1	*Cladonia*	*arbuscula*	J. Hoffman	210	North Carolina	USA	57 703	825×	OL989770
FEN-263	*Cladonia*	*coccifera*	E. Tripp	6067	North Carolina	USA	53 632	462×	OL989713
FEN-284	*Cladonia*	*didyma*	E. Tripp	6032	North Carolina	USA	52 819	264×	OL989719
FEN-470	*Cladonia*	*furcata*	E. Tripp	6423	Alabama	USA	59 822	409×	OL989724
FEN-508	*Cladonia*	*furcata*	J. Lendemer	49961	Alabama	USA	59 839	376×	OL989731
FEN-249	*Cladonia*	*grayi*	J. Lendemer	46385	North Carolina	USA	49 772	667×	OL989711
FEN-486	*Cladonia*	*leporina*	E. Tripp	6543	Alabama	USA	47 843	313×	OL989725
FEN-247	*Cladonia*	*macilenta*	J. Lendemer	46386	North Carolina	USA	46 499	500×	OL989710
FEN-250	*Cladonia*	*mateocyatha*	J. Lendemer	46387	North Carolina	USA	58 981	1313×	OL989712
SNG-RANG-2	*Cladonia*	*oricola*	J. Hoffman	438	Massachusetts	USA	54 684	2042×	OL989755
SNG-RANG-6	*Cladonia*	*oricola*	J. Hoffman	447	Massachusetts	USA	54 684	2151×	OL989756
FEN-420	*Cladonia*	*petrophila*	J. Lendemer	49138	Alabama	USA	53 100	179×	OL989723
FEN-445	*Cladonia*	*peziziformis*	E. Tripp	6281	Alabama	USA	45 312	—	NC_039132
FEN-267	*Cladonia*	*pyxidata*	E. Tripp	6047	North Carolina	USA	58 325	121×	OL989715
FEN-494	*Cladonia*	*rangiferina*	J. Lendemer	49896	Alabama	USA	58 220	454×	OL989730
CA-R-17	*Cladonia*	*rangiferina*	R. T. McMullin	19625	British Columbia	Canada	55 268	1408×	OL989694
SJN-807	*Cladonia*	*rangiferina*	J. Hoffman & Y. Ohmura	807	Hokkaido	Japan	55 109	4163×	OL989754
RJN-774	*Cladonia*	*rangiferina*	J. Hoffman & Y. Ohmura	774	Hokkaido	Japan	55 932	8268×	OL989749
RJN-772	*Cladonia*	*rangiferina*	J. Hoffman & Y. Ohmura	772	Hokkaido	Japan	55 975	3927×	OL989748
RJN-914	*Cladonia*	*rangiferina*	J. Hoffman & Y. Ohmura	914	Honshu	Japan	55 021	2956×	OL989750
RJN-938	*Cladonia*	*rangiferina*	J. Hoffman & Y. Ohmura	938	Honshu	Japan	55 106	2804×	OL989751
RJN-940	*Cladonia*	*rangiferina*	J. Hoffman & Y. Ohmura	940	Honshu	Japan	55 110	7639×	OL989753
R-BM2-1	*Cladonia*	*rangiferina*	J. Hoffman	681	Maine	USA	56 053	2802×	OL989737
R-BM2-4	*Cladonia*	*rangiferina*	J. Hoffman	680	Maine	USA	56 433	899×	OL989739
R-BM2-3	*Cladonia*	*rangiferina*	J. Hoffman	678	Maine	USA	57 427	3916×	OL989738
MI-R-4	*Cladonia*	*rangiferina*	J. Lendemer	44946	Michigan	USA	58 769	1150×	OL989736
WRG-RANG-6	*Cladonia*	*rangiferina*	J. Hoffman	235	North Carolina	USA	56 423	578×	OL989768
WRG-RANG-2	*Cladonia*	*rangiferina*	J. Hoffman	231	North Carolina	USA	56 426	898×	OL989767
WRM1-RANG-1	*Cladonia*	*rangiferina*	J. Hoffman	219	North Carolina	USA	56 449	274×	OL989771
FEN-201	*Cladonia*	*rangiferina*	J. Lendemer	46392	North Carolina	USA	58 935	152×	OL989709
R-NY-1	*Cladonia*	*rangiferina*	J. Franklin	GL1215A	New York	USA	56 432	2660×	OL989740
R-NY-2	*Cladonia*	*rangiferina*	J. Franklin	GL1215B	New York	USA	56 432	2049×	OL989741
R-NY-3	*Cladonia*	*rangiferina*	J. Franklin	GL1215	New York	USA	56 432	1523×	OL989742
CA-R-14	*Cladonia*	*rangiferina*	R. T. McMullin	16048	Newfoundland	Canada	56 203	995×	OL989693
CA-R-1	*Cladonia*	*rangiferina*	R. T. McMullin	17667	Nunavut	Canada	55 106	975×	OL989692
R-ON-3	*Cladonia*	*rangiferina*	R. T. McMullin	20379	Ontario	Canada	55 659	5082×	OL989743
R-ON-4	*Cladonia*	*rangiferina*	R. T. McMullin	20371	Ontario	Canada	56 484	4232×	OL989744
CA-R-6	*Cladonia*	*rangiferina*	R. T. McMullin	15494	Ontario	Canada	56 536	464×	OL989696
CA-R-9	*Cladonia*	*rangiferina*	R. T. McMullin	15935	Ontario	Canada	59 290	1830×	OL989698
CA-R-2	*Cladonia*	*rangiferina*	R. T. McMullin	14 331	Prince Edward Island	Canada	58 221	1429×	OL989695
CA-R-8	*Cladonia*	*rangiferina*	R. T. McMullin	12 115	Quebec	Canada	55 401	176×	OL989697
R-WA-2	*Cladonia*	*rangiferina*	J. Hoffman	704	Washington	USA	55 234	11 363×	OL989745
R-WA-3	*Cladonia*	*rangiferina*	J. Hoffman	702	Washington	USA	55 234	11 793×	OL989746
R-WA-4	*Cladonia*	*rangiferina*	J. Hoffman	703	Washington	USA	55 234	3585×	OL989747
FEN-491	*Cladonia*	*ravenelii*	J. Lendemer	49892	Alabama	USA	48 700	210×	OL989728
FEN-488	*Cladonia*	*squamosa*	E. Tripp	6547	Alabama	USA	49 980	528×	OL989726
FEN-280	*Cladonia*	*squamosa*	E. Tripp	6038	North Carolina	USA	55 771	401×	OL989718
FEN-278	*Cladonia*	*stipitata*	E. Tripp	6060	North Carolina	USA	60 062	102×	OL989717
FEN-265	*Cladonia*	*strepsilis*	E. Tripp	6049	North Carolina	USA	50 482	310×	OL989714
CA-ST-7	*Cladonia*	*stygia*	R. T. McMullin	7966	Nova Scotia	Canada	56 391	68×	OL989702
CA-ST-1	*Cladonia*	*stygia*	R. T. McMullin	15920	Ontario	Canada	58 388	691×	OL989699
CA-ST-5	*Cladonia*	*stygia*	R. T. McMullin	15589	Ontario	Canada	58 388	151×	OL989701
CA-ST-2	*Cladonia*	*stygia*	R. T. McMullin	15920	Ontario	Canada	58 446	646×	OL989700
SUBJ-736	*Cladonia*	*submitis*	J. Hoffman & Y. Ohmura	736	Hokkaido	Japan	51 791	4556×	OL989761
SUBJ-737	*Cladonia*	*submitis*	J. Hoffman & Y. Ohmura	737	Hokkaido	Japan	51 794	11 485×	OL989762
SUBJ-726	*Cladonia*	*submitis*	J. Hoffman & Y. Ohmura	726	Hokkaido	Japan	51 795	7226×	OL989760

(*Continued*)

Table 1. (Continued).

Isolate	Genus	Species	Collector	Collection number	Location	Country	Genome size (bp)	Coverage	Accession number
SUBJ-740	*Cladonia*	*submitis*	J. Hoffman & Y. Ohmura	740	Hokkaido	Japan	51 795	10 712×	OL989763
RJN-939	*Cladonia*	*submitis*	J. Hoffman & Y. Ohmura	939	Honshu	Japan	54 335	17 409×	OL989752
SUBJ-967	*Cladonia*	*submitis*	J. Hoffman & Y. Ohmura	967	Honshu	Japan	51 794	11 084×	OL989764
SUBJ-973	*Cladonia*	*submitis*	J. Hoffman & Y. Ohmura	973	Honshu	Japan	54 334	13 705×	OL989766
SNG-SMIT-9	*Cladonia*	*submitis*	J. Hoffman	451	Mass.	USA	56 879	1183×	OL989759
SNG-SMIT-5	*Cladonia*	*submitis*	J. Hoffman	445	Mass.	USA	56 895	2766×	OL989758
SNG-SMIT-11	*Cladonia*	*submitis*	J. Hoffman	454	Mass.	USA	56 895	2233×	OL989757
IB6-SMIT	*Cladonia*	*submitis*	J. Hoffman	357	New Jersey	USA	56 879	4088×	OL989734
IB4-SMIT	*Cladonia*	*submitis*	J. Hoffman	356	New Jersey	USA	56 889	238×	OL989733
IB7-SMIT	*Cladonia*	*submitis*	J. Hoffman	359	New Jersey	USA	56 900	827×	OL989735
IB3-SMIT	*Cladonia*	*submitis*	J. Hoffman	278	New Jersey	USA	56 901	261×	OL989732
FEN-493	*Cladonia*	*subtenuis*	J. Lendemer	49895	Alabama	USA	59 878	300×	OL989729
WRG-SP-2	*Cladonia*	*subtenuis*	J. Hoffman	248	North Carolina	USA	59 886	996×	OL989769
FEN-489	*Cladonia*	*uncialis*	E. Tripp	6550	Alabama	USA	63 607	347×	OL989727
FEN-277	*Cladonia*	*uncialis*	E. Tripp	6056	North Carolina	USA	63 608	319×	OL989716
FEN-200	*Cladonia*	*uncialis*	J. Lendemer	46391	North Carolina	USA	65 878	289×	OL989708
FEN-137	*Lepraria*	*caesiella*	J. Lendemer	45944	North Carolina	USA	45 176	293×	OL989703
FEN-145	*Lepraria*	*eburnea*	J. Lendemer	46116	North Carolina	USA	37 280	159×	OL989706
FEN-138	*Lepraria*	*finkii*	J. Lendemer	45962	North Carolina	USA	35 855	192×	OL989704
FEN-390	*Lepraria*	*normandinoides*	J. Lendemer	48841	Alabama	USA	41 379	43×	OL989722
FEN-49	*Lepraria*	*oxybapha*	J. Lendemer	46299	North Carolina	USA	40 221	—	KY348846.1
FEN-139	*Lepraria*	*vouauxii*	J. Lendemer	45983	North Carolina	USA	36 644	354×	OL989705
FEN-297	*Pilophorus*	*fibula*	E. Tripp	4988	North Carolina	USA	75 312	127×	OL989772
FEN-319	*Stereocaulon*	*dactylophyllum*	E. Tripp	5047	North Carolina	USA	57 569	398×	OL989720
FEN-176	*Stereocaulon*	*pileatum*	J. Lendemer	46616	North Carolina	USA	69 603	239×	OL989707

Isolate IDs noted in bold were sequenced using the Illumina Nova-Seq sequencer, whereas nonbold isolate IDs were sequenced with the Next-Seq sequencer.

standardized to the start of the COX1 gene, or until the sequence could not be built any further.

Prior to annotation, the genes annotated in the reference genomes were found to be irregular or, in some cases, incomplete. Thus, we revised these annotations prior to employing them as references to annotate our assembled genomes. Each coding sequence was aligned to examine the start codon, stop codon, and any introns present. Like many other fungi, lichen mitochondria are believed to use genetic code 4, for mold and protozoan mitochondria. When possible, genes were annotated to set the start codon as the one most conserved across the reference genomes, although priority was given to the standard start codon (ATG) when multiple possible start codons were found in proximity. Occasionally, an annotation for a coding region lacked a stop codon. In these cases, the 100 bp of sequence flanking the 3′ end of the annotation was searched for a stop codon within the reading frame. In cases where no unambiguous stop codon could be detected, the annotation was truncated at a consistent location across all genomes (e.g., COB, where the stop codon may be shared with COX2) or standardized to another annotation that did contain a stop codon (e.g., COX1).

When available, annotations without introns were used as a reference to edit and standardize annotations where introns were found. In the case of RPS3, sections of the sequence were previously inconsistently called as introns, but because the introns lacked internal stop codons, these regions were interpreted as indels. Lacking any cDNA data for rRNA subunits, these regions were standardized to the largest annotation. Finally, tRNAs were predicted using both tRNAscan-SE (Chan and Lowe 2019) and ARAGORN (Laslett and Canback 2004). Possible pseudogenes predicted by tRNAscan-SE were also noted.

Following revision of reference annotations, assembled mitochondrial genomes for all *Cladonia* and outgroup taxa were annotated using the Geneious annotation function. Annotation boundaries were checked using the open reading frame (ORF) finder in Geneious, identifying ORFs larger than or equal to 400 bp. Annotations were adjusted when they deviated from the boundaries of an overlapping identified ORF. In some samples, the stop codon for NAD3 could not be detected within 100 bp of the end of the annotation. This gene was left truncated. Any ORFs not matching an annotation were submitted to a BLASTp search. If the search returned multiple hits of the same type (e.g., COX1, HEGs, hypothetical protein), the ORF was annotated as showing similarity to that protein.

Identified HEGs were grouped by type (GIY-YIG or LAGLIDADG) then aligned using MAFFT in Geneious (Katoh and Standley 2013). HEGs were manually clustered into groups representing visually distinct assemblages of like-HEGs (e.g., LAGLIDADG group I). Each mitochondrial genome was then annotated with reference sequences in each group to determine the presence of HEGs in introns, even if its reading frame was interrupted by a stop codon.

Genome size analyses.—To compare genome size amongst newly assembled mitochondrial genomes, and with those previously published for other lichen and non-lichen ascomycetes, a data set of genome sizes was assembled from our data and from GenBank (SUPPLEMENTARY TABLE 2). Life strategy (lichen, plant pathogen or endophyte, animal or fungal pathogen, animal associate or gut flora, and saprophyte) was categorized for all species by reviewing relevant literature. Endophytes were grouped with plant pathogens here because every endophyte examined was noted in the literature as having a dual lifestyle as an opportunistic pathogen. Lichen taxa were additionally classified by growth form (i.e., crustose, foliose, fruticose, or polymorphic; leprose growth forms were treated as crustose, and squamulose growth forms as foliose) and by reproductive strategy (strictly sexual, dominant lichenized asexual with rare sexual reproduction, and strictly asexual without sexual reproduction). A Kruskal-Wallis test was used to determine whether mitochondrial genome size differed significantly across life strategies and lichens growth forms. Pairwise differences were tested with a post hoc Dunn test with a Holm family-wise error rate (FWER) method *P*-value adjustment.

Phylogenetic inference.—All 15 mitochondrial respiratory genes and both the large- and small-subunit rRNA regions (rrnL and rrnS, respectively) of assembled *Cladonia* and outgroup taxa (Cladoniaceae: *Pilophorus*; Stereocaulaceae: *Lepraria, Stereocaulon*) were extracted from the metagenomic data. Post annotation review, the published *C. peziziformis* mitochondrion (NC_039132) was added to this data set and the data aligned using MAFFT (Katoh and Standley 2013). Intronic data were removed from the alignments, leaving only exons or rRNA. The sequences were standardized at the ends and then concatenated. The maximum likelihood–based program RAxML (Stamatakis 2014) was used to infer a phylogeny of the mitochondrial gene suite. RAxML was run using 1000 rapid bootstraps and the GTR-gamma substitution model, which was supported by AICc (corrected Akaike information criterion) implemented in PartitionFinder2 (Lanfear et al. 2017).

Homing endonuclease ancestral state reconstruction.—The occurrence of individual HEGs in COX1 was manually tabulated, and the RAxML topology was used to estimate when the HEGs likely emerged. Parsimony-based ancestral state reconstruction (ASR) was conducted in Mesquite (Maddison and Maddison 2019) and run independently for each class of HEG. HEGs were categorized by whether stop codons had emerged in the sequence reading frame, and to what degree (i.e., whether the resulting sequence was reduced to less than 400 bp). These characters were mapped onto the ASR.

All representatives of each HEG group in *Cladonia* COX1 were aligned using MAFFT implemented in Geneious, with translations displayed using a uniform reading frame. In rare instances where HEGs were shared between *Cladonia* and outgroup taxa, outgroups were excluded from the alignment to examine only variation within *Cladonia*. Polymorphisms were scored for each alignment, recording the position in the codon where they occurred (first, second, or third position) to examine whether mutations were weighted toward one specific position. Indels were also scored as either triplet indels (indels in units of 3 bp) or non-triplet indels. Any distinct instance where an in-frame stop codon (TAA, TAG; mold, protozoan, and coelenterate mitochondrial genetic code 4) emerged upstream of the most distant recorded stop codon was also recorded.

RESULTS

Mitochondrial genome size: Interspecific variation.—In total, mitochondrial genomes from 73 samples representing 22 *Cladonia* species were assembled and circularized, along with nine outgroup species: one in Cladoniaceae (*Pilophorus fibula*) and eight in the closely related family Stereocaulacaeae (*Lepraria caesiella, L. eburnea, L. finkii, L. normandinoides, L. oxybapha, L. vouauxii, Stereocaulon dactylophyllum*, and *S. pileatum*; TABLE 1). Due to the diversity of sequencing platforms used to generate data for this study, genome coverage varied greatly, ranging between 43× and 13 705× (mean = 2233.0×, SD = 3206.9×). Mycobiont mitochondrial genomes represented between 0.06% and 5.04% of the total sequenced reads (mean = 1.33%, SD = 0.99%).

Mitochondrial genome size in *Cladonia* ranged from 45 312 bp (*C. peziziformis*) to 65 878 bp (*C. uncialis*), averaging 55 791.6 bp (SD = 3622.3 bp). Genomes were

made up of between 34.86% (*C. stygia* CA-ST-2) and 58.81% (*C. furcata* FEN-470) rRNA and respiratory genes. The amount of intergenic space ranged from 23 495 bp (*C. peziziformis*) to 38 228 bp (*C. stygia* CA-ST -2). *Stereocaulon* genomes were 57 569 bp (*S. dactylophyllum*) and 69 603 bp (*S. pileatum*) in size. Whereas *S. dactylophyllum* fell within the upper extent of genome sizes seen in *Cladonia, S. pileatum* was 3725 bp larger than the largest *Cladonia* mitochondrial genome. *Lepraria* genomes were consistently smaller than any of the *Cladonia* genomes, ranging from 35 855 bp (*L. finkii*) to 45 176 bp (*L. caesiella*).

To place the mitochondrial genome sizes of our primary study taxa within a broader evolutionary and functional context, we compiled data for an additional 50 lichen species and 199 non-lichen ascomycetes (SUPPLEMENTARY TABLE 1). We also scored several key traits for these taxa, namely, growth form and dominant reproductive mode for lichens and nutrition mode for both lichen and non-lichen taxa. Of the additional lichen taxa, the mitochondrial genomes of 31 were newly assembled with the genome assembly methods described here and 19 were previously published. The non-lichen fungal data were compiled from the National Center for Biotechnology Information (NCBI) database and included 228 separate samples from 199 species (85 genera), 167 of which were also studied by Pogoda et al. (2018). After Cladoniaceae, the second most deeply sampled lichen family was Parmeliaceae (26 complete mitochondrial genomes across 10 genera; SUPPLEMENTARY TABLE 2). Parmeliaceae exhibited greater mitochondrial genome size variation than Cladoniaceae or Stereocaulaceae (32 029 bp in *Imshaugia aleurites* to 108 024 bp in *Parmotrema stuppeum*), and Cladoniaceae had significantly smaller mitochondrial genomes than Parmeliaceae (two-sided Mann-Whitney *U*-test, $P < 0.05$, $U = -26\,380$; FIG. 1).

Mitochondrial genome size across all 81 lichen species from 28 genera and 15 families (135 total genomes) differed significantly across lichen growth forms (Kruskal-Wallis test, $\chi^2 = 31.416$, $df = 3$, $P < 0.05$; FIG. 2), with the mitochondrial genomes from crustose species significantly smaller than those of foliose and fruticose species (post hoc Dunn test, $P < 0.05$). Regarding reproductive strategies, the mitochondrial genomes of mixed-mode asexual+sexual species were significantly larger than those of strictly asexual or sexual species (Kruskal-Wallis test, $\chi^2 = 18.382$, $df = 2$, $P < 0.05$; post hoc Dunn test, $P < 0.05$). There was not a significant difference between strictly asexual and sexual species however until an outlier sample, *Leprocaulon disjuncta*, was removed from the strictly asexual data set (Kruskal-Wallis test, $\chi^2 = 21.856$, $df = 2$, $P < 0.05$; post hoc Dunn test, $P < 0.05$). Although scored as strictly asexual because sexual reproduction has never been observed in *L. disjuncta*, it belongs to a lineage that includes closely related species with mixed-mode reproduction (McCune and Rosentreter 2015).

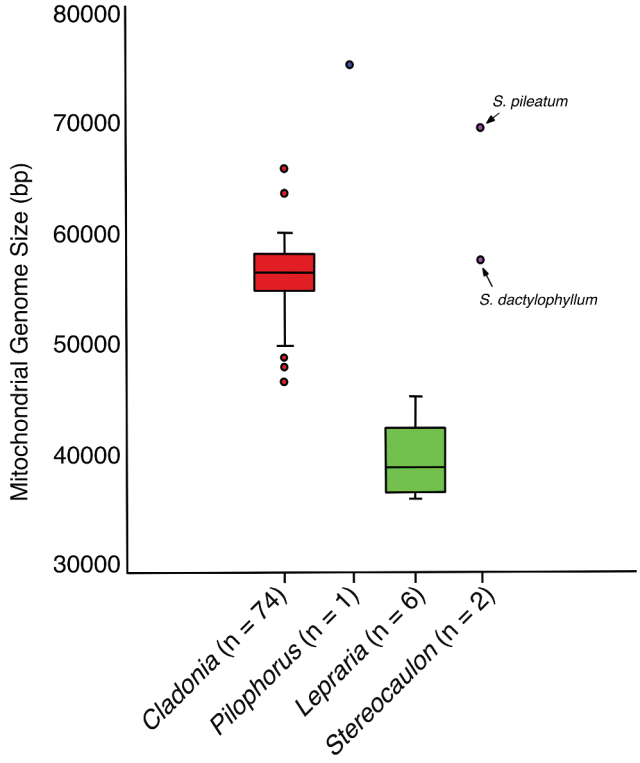
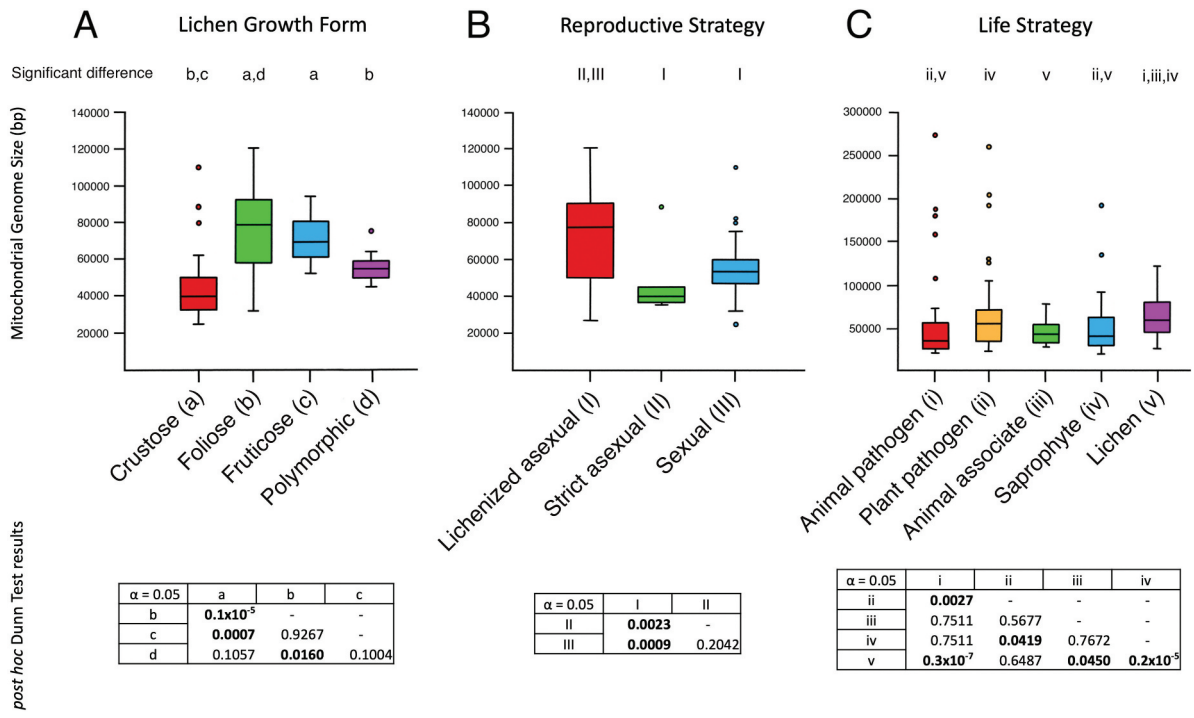


Figure 1. Mitochondrial genome sizes in *Cladonia, Pilophorus, Lepraria*, and *Stereocaulon*.

Non-lichenized ascomycetes exhibited far greater variation in mitochondrial genome size than lichenized fungi, ranging from 18 844 bp (*Haseniaspora uvarum*) to 272 497 bp (*Ophiocordyceps camponoti-floridani*). Of the 288 non-lichen genomes, 57 were endophytes and plant pathogens, 71 were animal or fungal pathogens, 80 were saprophytes or fungi of an otherwise free-living life strategy, and 19 were animal symbionts. Mitochondrial genome size varied significantly across fungal life strategies (Kruskal-Wallis test, $\chi^2 = 41.185$, $df = 4$, $P < 0.05$), with lichen genomes significantly larger than all other fungal lifestyles except for plant endophytes and pathogens (post hoc Dunn test, $P < 0.05$). Plant endophytes and pathogen mitochondrial genomes were also significantly larger than those of animal pathogens and saprophytes (post hoc Dunn test, $P < 0.05$).

Mitochondrial genome size: Intraspecific variation.—Of the 74 *Cladonia* mitochondrial genomes assembled here, 30 were from *C. rangiferina* and 14 were from *C. submitis*. Both species were sampled



α = 0.05	a	b	c
b	**0.1x10^{-5}**	-	-
c	**0.0007**	0.9267	-
d	0.1057	**0.0160**	0.1004

α = 0.05	I	II
II	**0.0023**	-
III	**0.0009**	0.2042

α = 0.05	i	ii	iii	iv
ii	**0.0027**	-	-	-
iii	0.7511	0.5677	-	-
iv	0.7511	**0.0419**	0.7672	-
v	**0.3x10^{-7}**	0.6487	**0.0450**	**0.2x10^{-5}**

Figure 2. Mitochondrial genome sizes among A) lichen growth forms (a = crustose, b = foliose and squamulose, c = fruticose, and d = polymorphic), B) lichen reproductive strategies (I = dominantly lichenized asexual, II = strictly asexual, and III = sexual), and C) fungal life strategies (i = animal pathogen, ii = plant pathogen or endophyte, iii = animal associate or gut flora, iv = saprophyte, and v = lichen). Post hoc Dunn test results are displayed in the tables below. Bold values indicate significance. Pairwise significant differences are listed above each column.

widely among their ranges in North America and East Asia. Due to the sampling depth, we examined intraspecific mitochondrial genome variation in these two species. There were 27 unique genotypes among the 30 *C. rangiferina* samples, whereas all 14 *C. submitis* samples represented unique genotypes. Genome size across *C. rangiferina* varied from 55 106 to 59 290 bp (4184 bp difference, mean = 56 351.8 bp, SD = 1212.2 bp), whereas the range was 51 791–57 826 bp across *C. submitis* (6035 bp difference, mean = 54 954.8 bp, SD = 2461.6 bp). Between 49.28% and 56.06% of *C. rangiferina* genomes and 50.10% to 52.53% of *C. submitis* genomes are composed of rRNA and the respiratory gene suite, leaving 24 798–30 071 bp in *C. rangiferina* and 24 587–28 349 bp in *C. submitis* as intergenic space.

Mitochondrial genome architecture.—The complete suite of respiratory genes and ribosomal RNA regions was recovered in all samples. Gene order was consistent within genera but varied among genera (FIG. 3). In comparing synteny among Cladoniaceae, *Cladonia* and *Pilophorus* gene order differed in the position of NAD1; whereas NAD1 occurred after COX1 in *Cladonia*, the gene was after COX2 in *Pilophorus*. *Lepraria*, which belongs to the Stereocaulaceae, exhibited the same gene order as *Pilophorus* in Cladoniaceae. However, *Stereocaulon* gene order deviated significantly from those of *Lepraria* and all members of Cladoniaceae: in addition to at least three instances of order rearrangement, all genes except for COX1 and NAD1 exhibited an inversion, reading in the opposite direction.

Within *Cladonia*, a consistent pattern of mitochondrial gene order was observed in all samples and species with few exceptions. First, a paralogous ATP9 gene was found in *C. ravenelii*, located between NAD3 and NAD4L, with a start and a stop codon, and not interrupted by any additional stop codons, appearing as an ORF (FIG. 4). A BLASTp search suggested strong similarity to known ATP9 (E value = 3×10^{-28}, percent identity = 92.31% match to *C. rangiferina* ATP9 [accession number NC_036309.1]). Second, within *S. pileatum* we found a sequence with high similarity (87.56%) to ATP9, located 278 bp downstream of the primary ATP9 copy. Although the sequence was highly similar to the primary ATP9, it lacked a terminal stop codon (FIG. 4). The nearest stop codon in the same reading frame was located 1614 bp downstream of where the gene would typically terminate. The sequence contained nine mutations separating it from its paralog, eight of which occurred in the last 18 bp of the 225 bp sequence.

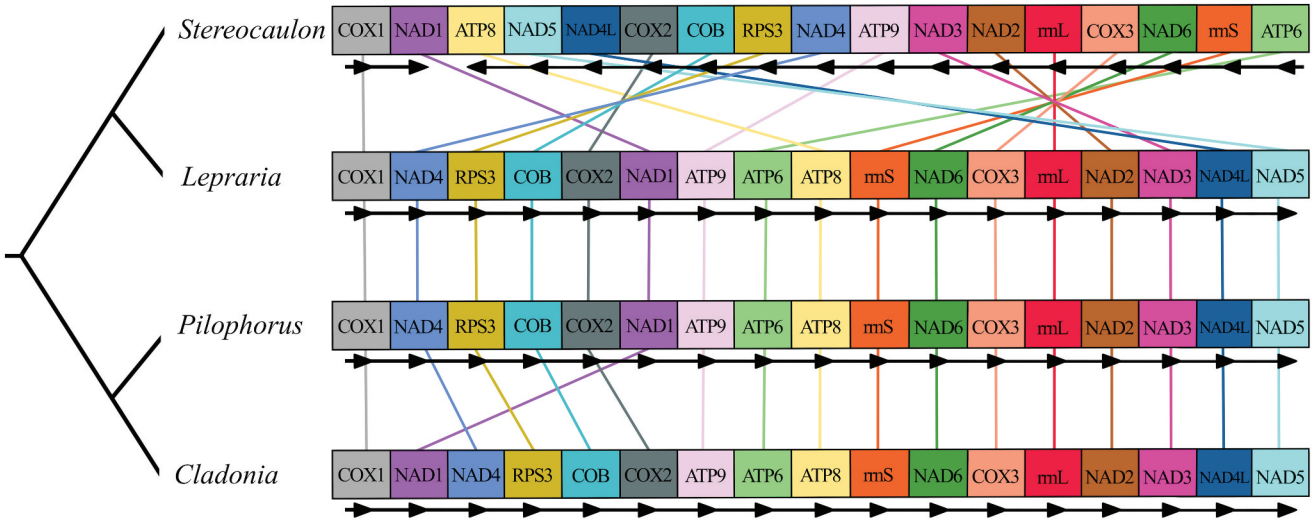


Figure 3. Mitochondrial gene order in *Cladonia, Pilophorus* (Cladoniaceae), *Lepraria*, and *Stereocaulon* (Stereocaulaceae). Arrows indicate reading direction.

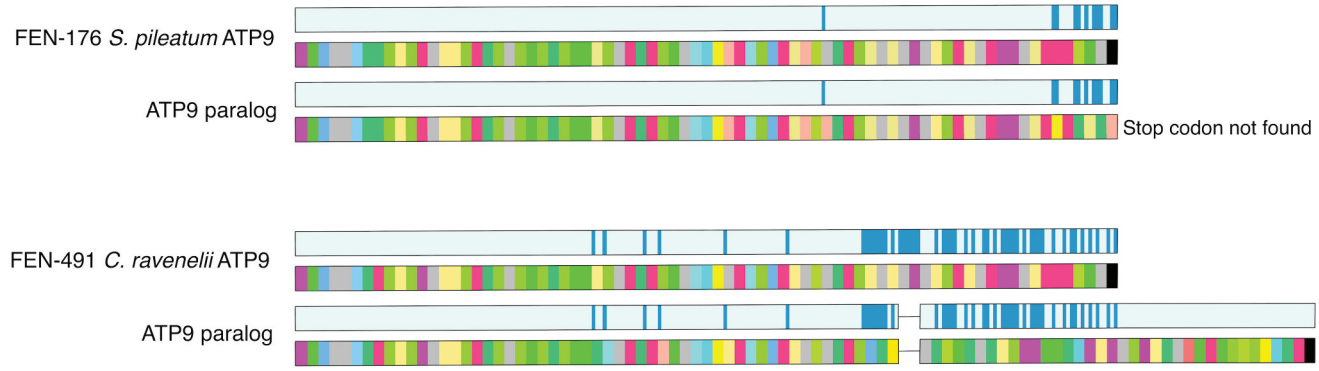


Figure 4. Mitochondrial ATP9 and gene paralog in *Stereocaulon Pileatum* and *Cladonia ravenelii*, depicted with nucleotide and amino acid sequences. Mutations are noted in dark blue.

We also found that *C. subtenuis, P. fibula, S. dactylophyllum*, and *S. pileatum* contained additional genes not associated with the mitochondrial respiratory gene suite. ORFs bearing strong similarity to DNA polymerase type B2 (DPO) were present in both *C. subtenuis* (between NAD3 and NAD4L) and *S. dactylophyllum* (between COX1 and NAD1). This gene was already noted in one *C. subtenuis* sample (FEN-493) by Birgham et al. (2018), which was reassembled and confirmed in this study. Our annotation of a second *C. subtenuis* sample (WRG-SP-2) identified a highly similar sequence (99.90% similarity to the uninterrupted ORF annotated in FEN-493), although the gene contained a stop codon (TAA) 288 codons into the 499-codon-long gene. ORFs bearing strong similarity to DNA-dependent RNA polymerase (RPO) were also found in *P. fibula* between COX1 and NAD4 and in *S. dactylophyllum* and *S. pileatum* between NAD1 and ATP8.

As has been observed in past studies (Birgham et al. 2018; Pogoda et al. 2018), the most variable mitochondrial gene regarding architecture was COX1. COX1 ranged from 1734 to 10 210 bp and had anywhere from zero to six determinable introns. When COX1 introns were present, intron size ranged from 937 to 8378 total bp, constituting between 16.85% and 63.95% of the gene (mean = 30.39%, SD = 8.88%). The fewest COX1 introns were observed in *C. peziziformis* (no introns), whereas the most were in *C. furcata, C. petrophila*, and *C. stipitata* (six introns). In 37 of 74 *Cladonia* samples, the COX1 stop codon could not be determined and was annotated as truncated, such that the upper limit to the number of introns remains uncertain.

Phylogenetic inference.—To examine the evolutionary history of HEGs in *Cladonia*, we first inferred a phylogeny from the complete suite of mitochondrial

respiratory gene exons and ribosomal rRNA. *Pilophorus* was included because it is a known early diverging lineage in Cladoniaceae (Stenroos et al. 2019), and representatives of Stereocaulaceae (*Leprara, Stereocaulon*) were included as an outgroup based on the well-established sister relationship between that family and Cladoniaceae (Midalikowska et al. 2006). The maximum likelihood phylogeny we recovered was largely topologically congruent with that inferred by Stenroos et al. (2019) from five nuclear loci. All major clades and subclades delimited by Stenroos et al. (2019) were recovered and supported in this study (FIG. 5): clade Arbusculae represented by *C. submitis* and *C. arbuscula* was strongly supported (bootstrap support [BP] = 100); clade Crustaceae represented by *C. rangiferina, C. stygia, C. oricola*, and *C. subtenuis* was strongly supported (BP = 98); clade Erythrocarpae represented by *C. coccifera, C. didyma, C. leporina, C. macilenta, C. ravenelii* was strongly supported (BP = 100); clade Perviae represented by *C. strepsilis* and *C. squamosa* was strongly supported (BP = 100); and clade Unciales represented by *C. uncialis* was supported (BP = 75).

In addition to the clades outlined above, clade Cladonia of Stenroos et al. (2019) was recovered as monophyletic with strong support (BP = 100) and included subclades Apodocarpae (*C. apodocarpa, C. petrophila*), Ascyphiferae (*C. furcata, C. peziziformis, C. stipitata*), Cladonia (*C. mateocyatha*), and Graciles (*C. grayi, C. pyxidata*). Each of those four subclades within clade Cladonia was recovered as monophyletic with strong support (BP = 100). Although the phylogeny inferred here recovered all the major groups within *Cladonia* that had been identified in previous evolutionary studies, the relationships among these clades differed slightly from Stenroos et al. (2019): namely, clades Erythrocarpae and Perviae were here recovered as sister to clade Crustaceae rather than Arbuscula. Moreover, whereas clades Arbuscula and Crustaceae were recovered as sister by Stenroos et al. (2019), they were here recovered as more distantly related, separated by the clades Uncialis (BP = 75), Erythrocarpiae (BP = 100), and Perviae (BP = 98).

Homing endonucleases: Interspecific variation.— Homing endonuclease genes were detected in all samples. BLASTp searches of intraintron ORFs across all samples revealed HEG-like sequences (i.e., ORFs that showed a high similarity to known HEG sequences) in COB, COX1, NAD1, and NAD5. In total, 11 unique LAGLIDADG HEGs and three GIY-YIG HEGs were identified across all *Cladonia* species. Both types of HEGs exhibited high site fidelity, with each HEG group consistently restricted to one intron location, in only one gene. Four LAGLIDADG and two GIY-YIG HEGs were found in *P. fibula*, located in the COB, COX1, and COX3 genes and in both rrnL and rrnS rRNAs. HEGs were comparatively scarce in *Lepraria*, where all species had one LAGLIDADG HEG in the intron of NAD5. Two ORFs were also found in *L. caesiella* intergenic space, resembling LAGLIDADG and GIY-YIG HEGs between COX3 and rrnL and between rrnL and NAD2, respectively.

Of all HEGs documented, six LAGLIDADG groups (which we designated LAGLIDADG groups I–VI) and two GIY-YIG groups (which we designated GIY-YIG groups I–II) were unique to COX1. Two LAGLIDADG HEGs (groups III and VI) were also represented in *P. fibula* (group VI) and *S. pileatum* (groups III and VI). COX1 contained the most variability in endonuclease occurrence across samples, with samples bearing between one and six LAGLIDADG or GIY-YIG HEG-like sequences. Although introns usually contained zero to one endonuclease, some introns contained two. Non-HEG intraintron ORFs were rare, but two hypothetical proteins were identified in one *C. rangiferina* sample (CA-R-2) and all three *C. uncialis* samples. Whereas the ORF in CA-R-2 could not be identified via BLASTp, those in *C. uncialis* COX1 intronic space were identified as a DUF3810 domain–containing protein.

Of the eight COX1 HEGs found in *Cladonia*, seven exhibited between 70 and 199 substitution mutations (mean = 99.86, SD = 47.28) and between zero and eight indels (TABLE 2). LAGLIDADG group IV HEG was highly similar across the six taxa in which they were found, with only a single mutation observed. In these seven HEGs, the position of substitution mutations varied considerably. The representation of third-position mutations fluctuated from 20.00% of the total in GIY-YIG group II to 62.82% in LAGLIDADG group VI (mean = 41.02%, SD = 12.22%). Excluding LAGLIDAG group IV, synonymous mutations represented between 34.7% and 57.7% of all mutations. In both GIY-YIG HEGs and half of LAGLIDADG HEGs (groups I, II, and VI), frameshift mutations were present in at least one taxon. In all cases, non-triplet indels interrupted sequence translation at some point with a stop codon. All HEGs except for LAGLIDADG groups III and IV were interrupted with an early stop codon in at least one taxon.

Homing endonucleases: Intraspecific variation.— As was the case among *Cladonia* species, considerable

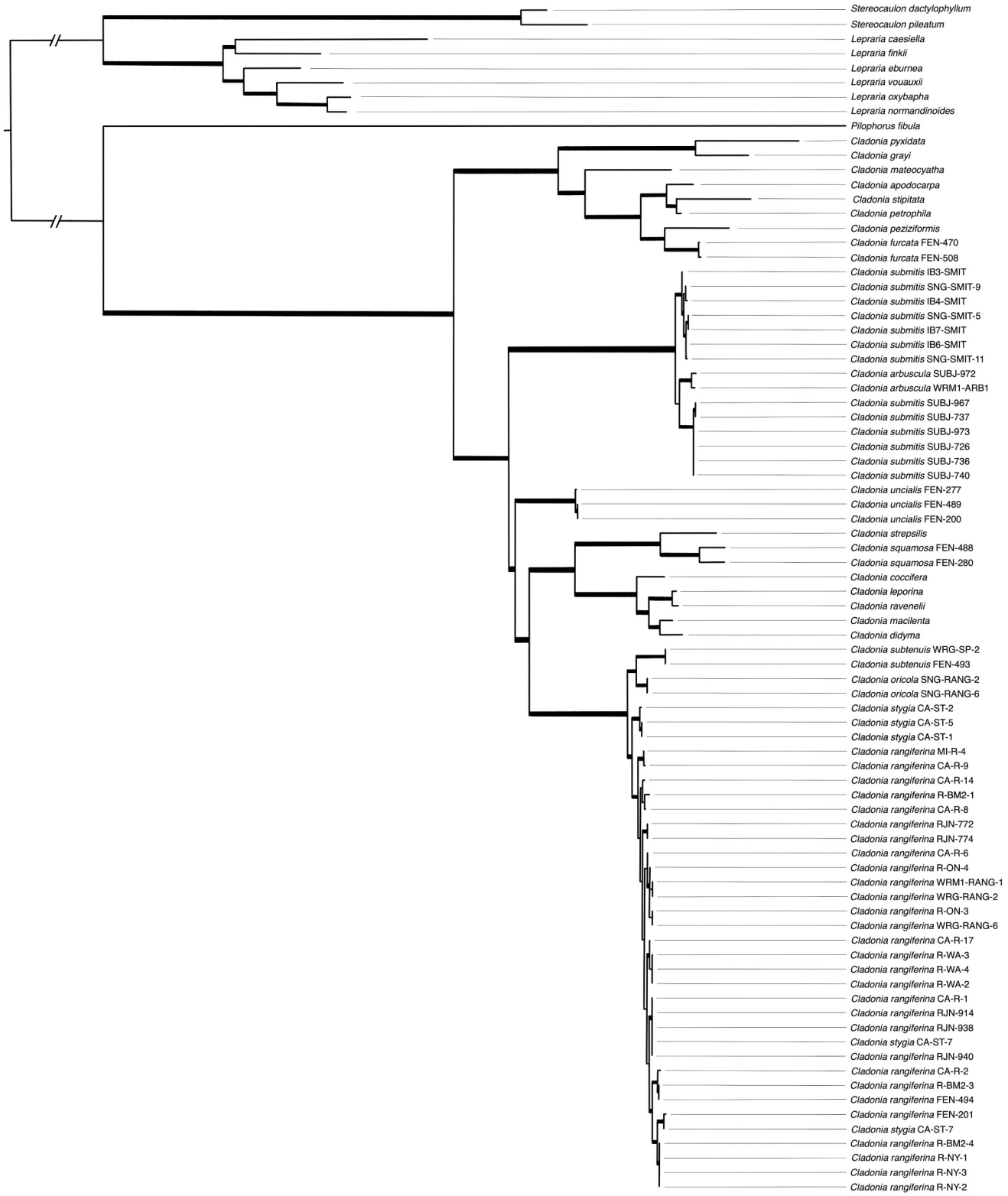


Figure 5. RAxML phylogeny of the respiratory gene suite and rRNA genes of the mitochondria of *Cladonia* and outgroup taxa. Branches with bootstrap support of 75% or greater are bold.

variation in intron and HEG presence was also observed within *C. rangiferina* and *C. submitis*. One of the two GIY-YIG HEGs and five of six LAGLIDADG HEGs that occurred in *Cladonia* were found in *C. rangiferina*, whereas no GIY-YIG HEGs and two LAGLIDADG HEGs were found in *C. submitis*. In *C. rangiferina*, COX1 had three to four introns, although the gene was truncated in 22 samples (73%), with at least one unidentified intron present

Table 2. Mutations, indels, and stop codons found in the GIY-YIG and LAGLIDADG homing endonuclease genes (HEGs) in *Cladonia*.

		Point mutations						Indels			In-frame stop codons	
HEG type	Group	1st position	2nd position	3rd position	Total	% in 3rd position	% synonymous	Non-triplet indels	Triplet indels	Total	Stops	Affected samples
GIY-YIG	I	31	16	23	70	32.9	54.3	2	3	5	1	1
	II	22	12	19	53	35.8	24.5	2	1	3	2	3
LAGLIDADG	I	33	31	49	113	43.4	41.6	4	4	8	5	9
	II	23	21	28	72	38.9	34.7	1	1	2	1	4
	III	39	33	50	122	41.0	35.2	0	0	0	1	3
	IV	1	0	0	1	—	0	0	0	0	0	0
	V	56	47	96	199	48.2	57.3	0	3	3	0	0
	VI	14	15	49	78	62.8	57.7	1	1	2	1	1

toward the 3′ end of the gene. In *C. submitis*, COX1 had one to three introns, with the gene truncated in nine samples (64%). *Cladonia rangiferina* COX1 contained two to four HEGs, exhibiting one of the two identified GIY-YIG types and five of the six LAGLIDADG types. *Cladonia submitis* COX1 contained fewer HEGs, exhibiting either one or two LAGLIDADG HEGs. Of all HEGs, LAGLIDADG group IV was exclusive to *C. rangiferina*, found in only six samples (FIG. 3).

Homing endonucleases: Ancestral state reconstruction.—Parsimony-based ancestral state reconstructions (ASRs) of each distinct HEG group in COX1 revealed very different histories of inheritance (SUPPLEMENTARY FIGS. 1A–F). One LAGLIDADG HEG (group IV) and one GIY-YIG HEG (group I) were each restricted to one clade and predicted to have had a single origin in *Cladonia*. The other six COX1 HEGs were paraphyletic, with all but LAGLIDADG group III predicted to have multiple origins in *Cladonia*. Three LAGLIDADG HEGs (groups I, III, and VI) were much more widespread than other HEGs, occurring in more than 50% of examined *Cladonia* species. In group I, III, and VI LAGLIDADG and group I GIY-YIG HEGs, at least one instance of HEG loss was also inferred.

DISCUSSION

This study presents the first comparative analysis of intraspecific and interspecific mitochondrial genome variation within a single lineage of lichen-forming fungi (i.e., the clade formed by Cladoniaceae and Stereocaulaceae; Miadlikowska et al. 2006) and focuses on the iconic, ecologically important macrolichen genus *Cladonia*. The 74 samples belonging to 21 *Cladonia* species and one *Pilophorus* species studied here render *Cladonia* the best-sampled lichen genus and family with respect to assembled mitochondrial genomes. (Parmelicaeae, a highly speciose lichen family, is represented by mitochondrial genomes from 27 total species from 10 genera, of which 14 are newly published here).

Mitochondrial genome size: Interspecific variation.—Mitochondrial genome size was significantly smaller in Cladoniaceae and Stereocaulaceae than in Parmeliaceae and exhibited a smaller size range. The difference in size may be related to the greater representation of Parmeliaceae genera. Sampling other genera in Cladoniaceae and Stereocaulaceae, and sampling more robustly in the genera studied here, will provide greater insight into whether the variation is related to the number of genera sampled.

Cladonia mitochondria exhibited a near 20 000 bp range in size, from 46 499 to 65 878 bp. Whereas the *P. fibula* mitochondrion was somewhat comparable in size, and *Stereocaulon* was toward the larger end of this range, *Lepraria* genomes were between 1323 and 10 644 bp smaller than the smallest assembled *Cladonia* genome. *Lepraria* species had few introns, exhibiting far less intronic space compared with *Cladonia, Pilophorus*, and *Stereocaulon*. *Cladonia* and *Lepraria* differ considerably in aspects of morphology and reproductive strategy; *Cladonia* species form large, morphologically complex thalli, and these vary greatly across its constituent clades (Ahti 2000; Brodo et al. 2001). By contrast, *Lepraria* species have highly reduced, simplified thalli that consist primarily of diffuse bundles formed from hyphae wrapped around algal cells that adhere to a substrate (Lendemer 2013). *Cladonia* species utilize a diversity of reproductive modes in individual thalli, often combining sexual, asexual, and vegetative reproduction (Seymour et al. 2005). *Lepraria*, however, is persistently sterile; neither sexual reproductive structures nor sexual reproduction has ever been documented in the

lineage (Lendemer 2013). The differences between Cladoniaceae and *Lepraria* mitochondrial genome sizes and intron occurrence may reflect different selective pressures related to the differences in morphological complexity, reproductive strategies, or both.

When compared across lichen genera, using genomes generated in this study and those available on GenBank, mitochondrial genome size differed significantly across lichen growth forms. Crustose lichens, which have the most limited range of morphological variation, had significantly smaller mitochondrial genomes than those of morphologically more complex fruticose and foliose lichens. There was not a significant difference between crustose and polymorphic lichens, which is surprising given that species with polymorphic morphologies combine multiple growth forms in a single dimorphic thallus (Ahti 1961). It is possible that this could be due to a phylogenetic effect, where a correlation between growth form and genome size could be explained by relatedness. However, although growth form is usually consistent within genera, there is often variation among even closely related genera (Arup et al. 2013; Lendemer and Hodkinson 2013; Tehler and Irestedt 2007). A similar assessment of mitochondrial genome size to other genome characteristics by Pogoda et al. (2018) found that their results remained significant even after correcting for phylogenetic relatedness. A broader study incorporating intensive sampling within a greater range of families is needed.

Mitochondrial genome size was also significantly related to reproductive strategy, with species utilizing mixed asexual+sexual modes (n = 38) possessing significantly larger genomes than either sexual (n = 41) or strictly asexual (n = 7) species. Species with such mixed modes also exhibited the greatest variation in genome size (FIG. 2B), with strictly sexual or asexual species exhibiting far more constrained sizes. Reproductive flexibility present in mixed-mode species may be a strategy that encourages experimentation in genome content, which translates to a greater range in genome sizes. Alternatively, fixed reproductive modes may constrain genome size to some degree. It is possible that *Lepraria* is constrained in mitochondrial genome size due to factors other than reproductive strategy. However, our sample sizes in terms of represented genera are low, particularly for strictly asexual lichens, so there is a need for increased targeted sampling of strictly asexual taxa and their sisters.

In a broader context of fungi across Ascomycota, mitochondrial genome size is significantly larger in lichens than in most other fungal lifestyles. Like other obligate symbionts, lichens cannot develop to maturity and reproduce in the absence of their photobiont (Nash 1996). Long-standing coevolutionary bonds between species such as those in lichens theoretically drive genomes toward streamlining, a process by which symbionts partition roles and eliminate redundant processes (Khachane et al. 2007; McCutcheon and Von Dohlen 2011; Morris et al. 2012). Genome streamlining frequently results in simplified or lost genes, which suggests that the resulting genome size should be smaller in obligate symbionts than in facultative or free-living organisms. However, total genome size does not necessarily relate to streamlining (Lee and Marx 2012); although many bacteria do exhibit size reductions tied to streamlining—and indeed this phenomenon accounts for the evolution of mitochondria as ancient endosymbionts (Adams and Palmer 2003; Khachane et al. 2007; Morris et al. 2012)—several fungi and some bacteria exhibit genome inflation instead (Martin et al. 2010; Spanu et al. 2010). Although we did not find reduced mitochondrial genome sizes in lichens, other studies have demonstrated some evidence for streamlining in certain species (Pogoda et al. 2019).

Mitochondrial genome size: Intraspecific variation.—Although exhibiting less variation than was seen across *Cladonia*, mitochondrial genome size still varied by 4269 bp within *C. rangiferina* and 6035 bp within *C. submitis*. Intergenic space varied by 5273 bp in *C. rangiferina* and 3762 bp in *C. submitis*, but much of this size difference is jointly due to the variation in intron occurrence. In *C. rangiferina*, smaller genomes are widespread across East Asia and North America, although some larger genomes did occur on the east Atlantic Coast of North America. In *C. submitis*, mitochondrial genomes from East Asian samples were consistently smaller than those from eastern North America (unpaired *t*-test, $P < 0.01$, $t = 9.3289$). This could reflect disjunction and hence strong geographic isolation of the two populations (Ahti 1961; Hoffman et al. 2020). That we recovered *C. submitis* mitochondrial genomes in two distinct, geographically restricted clades further supports this hypothesis.

Mitochondrial genome architecture.—A comparison of gene order among *Cladonia* revealed a paralogous ATP9 in *C. ravenelii*. The duplication is interesting in the context of its loss in numerous other lichen lineages (Pogoda et al. 2018). In mitochondria, the ATP9 encoded protein is a critical part of the ATP synthase

enzyme, with multiple copies forming a ring that rotates during proton transport (Bietenhader et al. 2012). Its activation promotes the critical ATP-producing capability of ATP synthase, which is likely why it is so conserved in *Cladonia*; although nucleotide polymorphisms were present across all samples, none caused any differentiation in their corresponding codons, including in the first copy of ATP9 in *C. ravenelii*. However, the duplicated ATP9 in *C. ravenelii* had many variations that change its translation, including several large insertions (FIG. 3). One explanation for this divergence is that the duplicated gene is not translated due to factors not apparent in the mitochondrial genome sequence and has accumulated mutations that, although not resulting in an interrupting stop codon in the sequence, left the sequence functional, but not translated. Effectively the result is a pseudogene, a phenomenon that occurs often in genomic evolution (Balakirev and Ayala 2003; Bensasson et al. 2001; van der Burgt et al. 2014). This hypothesis is also a likely explanation for the duplicate ATP9 seen in *S. pileatum*, which lacked a discernable stop codon.

An alternative hypothesis for the paralogous ATP9 in *C. ravenelii* is neofunctionalization: that the paralogous gene is still translated but serves an altered function. Likewise, it is also possible that the paralogous ATP9 in *S. pileatum* is also translated, perhaps containing an undetected intron toward the 3′ end of the sequence. Duplications of respiratory proteins are not rare in fungi (Marcet-Houben et al. 2009), and there are instances where a paralogous gene accrues mutations and develops a specialized function. For example, *Saccharomyces cerevisiae* has two variants of COX5, one expressed under aerobic conditions and the other under anaerobic conditions (Hodge et al. 1989). Similarly, paralogous ADP/ATP carriers in *Yarrowia lipolytica* are differentially expressed under aerobic and anaerobic conditions (Mentel et al. 2005). The duplication and differentiation of ATP9 in *C. ravenelii* could be one such gene specialization event, wherein the second function has yet to be identified. It is worth noting that although such duplications are common in fungal mitochondria, this is the first documented case in lichens. Additional sampling of *C. ravenelii* is required to further study this phenomenon.

The other notable difference in gene content was the occurrence of DNA polymerase (DPO) and RNA polymerase (RPO) in several species across multiple genera. The occurrences of DPO and RPO are the result of an integration of a partial or entire plasmid containing both genes into the mitochondrial genome, an event often reported in fungi (Formighieri et al. 2008; Griffiths 1995; Nadimi et al. 2015). Lichens in multiple genera are known to contain DPO or RPO (Pogoda et al. 2018; Xavier 2011). The presence of DPO in *C. subtenuis* was previously reported by Birgham et al. (2018) and is evidence that, like other fungi, mitochondrial plasmids likely play a role in the organization and transformation of lichen genome architecture. However, the additional sample of *C. subtenuis* examined in this study contained a nonfunctional DPO, interrupted by a single stop codon. The sporadic occurrence of DPO and RPO in lichen mitochondrial genomes further suggests that the genes serve little or no function once inserted into the mitochondrion. In the Stereocaulaceae, which is sister to Cladoniaceae, DPO was also found in *S. dactylophyllum*, and RPO in *P. fibula, S. dactylophyllum*, and *S. pileatum*. In *S. dactylophyllum*, DPO and RPO were separated by NAD1, which could be explained by separate plasmids integration events or by transposition of NAD1 between the two plasmid genes after their insertion.

Homing endonucleases: Interspecific variation.—The abundance of LAGLIDADG and GIY-YIG HEGs in lichens, and more specifically *Cladonia*, has been noted in recent studies (Birgham et al. 2018; Pogoda et al. 2018, 2019). Pogoda et al. (2018) found that GIY-YIG HEGs were more abundant than LAGLIDADG HEGs across an evolutionarily broad sampling of lichens. However, within *Cladonia*, we found that LAGLIDADG HEGs were far more common and diverse than GIY-YIG HEGs. HEGs are a common selfish genetic element that occurs widely in genomes across animals (Barzel et al. 2011; Chan et al. 2011; Fukami et al. 2007), plants (Brouard et al. 2010; Del Hoyo et al. 2018; Haugen et al. 1999), and particularly fungi (Belfort and Roberts 1997; Doolittle 1993; Megarioti and Kouvelis 2020; Reeb et al. 2007). Two other distinct classes of HEGs (HNH and His-Cis box) have been described, and whereas HNH, LAGLIDADG, and GIY-YIG HEGs have been observed in other fungi (Belfort and Roberts 1997; Megarioti and Kouvelis 2020), only the latter two have been found in lichens (Birgham et al. 2018; Pogoda et al. 2018, 2019). Our study is consistent with this.

The literature on HEGs is somewhat divided on the nature of these transgenomic sequences. Some studies have suggested that HEGs are parasitic genomic elements with "life cycles" of their own; HEGs insert into conserved elements of genes such as COX1, potentially invoking a cost on the host gene and organism (Goddard and Burt 1999; Gogarten and Hilario 2006; Megarioti and Kouvelis 2020). Upon insertion as an intron, the HEG persists until it is fixed in the population, after which it accrues mutations and loses function (Gogarten and

Hilario 2006; Swithers et al. 2009). By inhabiting an intron flanked by conserved elements, HEGs may also be possibly sheltered from some deleterious mutations that could erode the endonuclease function or eliminate the intron. This perspective is the dominant one in lichens, with HEGs discussed mostly as parasitic elements in the limited lichen literature (Pogoda et al. 2019).

Other studies hold the alternate perspective that HEGs may instead be mutualistic elements, at least in some cases (Hafez and Hausner 2012; Stoddard 2005). HEGs introduce new alleles upon introduction (Basse 2010) that may be advantageous to the host. Additionally, these mobile elements are strongly associated and coevolved with group I and II introns (Megarioti and Kouvelis 2020), which are in turn hypothesized to have coevolved mutualisms with host genes (Edgell et al. 2011). Introns are a well-known source of potential functional diversity for proteins, creating potential for alternative splicing (Kelemen et al. 2013; Kim et al. 2008; Syed et al. 2012). By introducing introns into a gene, HEGs may also introduce opportunities for translational innovation. There are instances of specific HEGs escaping the cycle of invasion and elimination by adopting a function valuable to the host (Stoddard 2005). Whereas some HEGs have developed a splicing function for the host intron (Lambowitz et al. 1999), others have taken on more specialized roles, such as cleaving the MAT plasmid in *Saccharomyces cerevisiae* (Jin et al. 1997).

We found a clear dichotomy in our focal taxa (Cladoniaceae and Stereocaulaceae) between genera with abundant HEGs (*Cladonia, Pilophorus*, and *Stereocaulon*) and *Lepraria*, which was comparatively HEG-poor. Across *Cladonia, Pilophorus*, and *Stereocaulon*, only a single sample (*C. peziziformis*) lacked any HEGs in COX1. Species in these genera contained from one to ten HEGs across the mitochondrial genome (mean = 5.13, SD = 2.22), whereas *Lepraria* contained between one and three (and two of the three HEGs in *L. caesiella* were in intergenic space). *Lepraria* species consistently had only two or three introns, which reflects the strong association between HEGs and intron presence. The reduction or loss of introns may be additional evidence of evolutionary pressure toward streamlining in *Lepraria*, which is highly morphologically reduced in comparison with the complex morphologies of *Cladonia, Pilophorus*, and *Stereocaulon*. If this is the case, the trend toward reduction could explain the paucity of HEGs in *Lepraria*, as any introns created by their occurrence could potentially be more costly than in other genera.

In this way, HEGs could act as parasites and mutualists depending on the evolutionary pressures of the host taxon. When introns are selected against, HEGs invoke a cost on their host genes by introducing disadvantageous introns. When genomic experimentation is evolutionarily favored, or less subject to strong selective pressure, HEGs could function mutualistically as a source of introns or genetic variation. *Cladonia* and *Lepraria* could be an ideal system to investigate function of endonucleases in obligately symbiotic organisms, particularly through a cDNA study, in which the transcripts of genes with and without introns would be sequenced to determine whether genes with HEG introns undergo alternative splicing.

Our study suggests that HEG inheritance is more complicated than that of the lichen mitochondrion. Ancestral state reconstructions inferred multiple evolutionary histories for COX1 HEGs, which sometimes deviated from the relationships in the phylogeny. These ASRs inferred multiple instances of insertion for several HEGs. This is similar to Goddard and Burt (1999), who observed multiple patterns of intron and HEG occurrence in *S. cerevisiae* mitochondrial rrnL genes, which did not coincide with phylogenetic relationships. Those authors concluded this was the result of horizontal gene transfer between distinct *S. cerevisiae* strains. Horizontal gene transfer also likely explains our results, although it likely involves another genome abundantly present in the lichen itself (e.g., the mycobiont nuclear genome, or possibly photobiont nuclear or organellar genomes). Horizontal gene transfer has already been observed between the nuclear and mitochondrial mycobiont genomes (Pogoda et al. 2018; Tagirdzhanova et al. 2021). Additionally, although exchange with photobiont genomes has yet to be observed, many fungi have experienced horizontal gene transfer with bacteria (Fitzpatrick 2012; Schmitt and Lumbsch 2009) and other fungi (Fitzpatrick 2012) and even plants (Nikolaidis et al. 2014). Thus, it is possible that such exchanges could occur between lichen bionts.

Despite evidence for horizontal gene transfer, no single HEG was found in more than one location in the genome, even across species. This strong fidelity is illustrated by the subset of ASRs in which an HEG group was inferred to have arisen independently multiple times in *Cladonia* yet always occurred at the same location in COX1. This demonstrates the strength of the HEG homing ability. Like restriction enzyme affiliation to restriction sites, HEGs have regions with an affinity for specific target sites (Stoddard 2005). HEG target sites are far larger than those of restriction enzymes, ranging from 14 to 44 bp (Arbuthnot 2015; Stoddard 2005). However, HEGs also exhibit more flexibility in binding specificity and thus can insert into more variable sites

(Hafez and Hausner 2012). This may include similar regions in the same genome, as well as the same region across individuals or species. It is this variability that may explain the inference of multiple separate insertion events in the same region of a gene.

The accrual of mutations within COX1 HEGs varied considerably: some had nearly equal ratios of transformation mutations in each codon position, whereas others had a strong bias toward the third position. The latter position bias may indicate selective pressure toward conservation of function, as third-position mutations often code for the same amino acid (Crick 1966; Yarus 2021). It may be that some HEGs we found in still retain their transposon-like function. HEGs are known to self-replicate both independently and through proximal translation (Hafez and Hausner 2012; Sethuraman et al. 2009), and these HEGs may still be capable of transposing and inserting into a new location. However, there is also evidence of endonuclease degradation in the mitochondria we studied. All but three COX1 HEGs had non-triplet indels in at least one sample, and all but two had at least one instance of a stop codon interruption. These frameshift mutations and stop codons indicate that HEGs do not retain function, at least in some taxa, and instead accrue mutations regardless of their effect on the gene.

One possible explanation for the apparent contradiction between conservation and interruption is the HEG life cycle. Originating elsewhere in the lichen genome, the HEG may retain conservation of function, preserving capacity to insert into specific genes. Upon insertion into a mitochondrial gene, however, it may be incapable of transportation to a new position, subsequently losing function it had outside of the mitochondrion and undergoing degradation by accruing deleterious mutations.

Homing endonucleases: Intraspecific variation.— Homing endonuclease genes also varied within the two taxa we studied, *C. rangiferina* and *C. submitis*. Between two and four COX1 HEGs were observed in *C. rangiferina*, whereas one or two were observed in *C. submitis*. There was also one HEG (LAGLIDADG group IV) that was unique to *C. rangiferina* and found only in five samples (SUPPLEMENTARY FIG. 1F). The emergence of this LAGLIDADG HEG may be a recent evolutionary event. However, additional interspecific and intraspecific sampling is needed to confirm this.

The interspecific mitochondrial genomic and HEG diversity of *C. rangiferina* and *C. submitis* found in this study are a likely representative of what could be found in other *Cladonia* species if studied to a similar degree. Nuclear genomic diversity strongly relates to effective population size (N_e; Hague and Routman 2016), although this relationship does not necessarily extend to mitochondrial diversity, at least in animals (Bazin et al. 2006; Meiklejohn et al. 2007). This phenomenon has been tied to genetic draft, a process by which advantageous alleles under strong selection become fixed and facilitate a sweep of allele fixations at nearby linked loci (Gillespie 2000, 2001). The effect is strong in mitochondria due to its typically uniparental inheritance and lack of recombination—a trait consistent in many, though not all fungi (Wilson and Xu 2012). However, due to their origins from outside of the organelle, the occurrence of HEGs might not be subject to the same genetic draft effect. Hence, HEGs may have the same relationship with N_e that is present in nuclear DNA. Under this hypothesis, species with large populations or that are widely distributed (e.g., *C. rangiferina*; Culberson 1972) would have more unique HEGs, whereas restricted species with smaller populations (e.g., *C. submitis*; Ahti 1967; Hoffman et al. 2020) would have less HEG diversity. Additional population-level sampling of multiple species is needed to test this hypothesis.

CONCLUSION

This study presents the largest assessment of mitochondrial genome variation within a lichen genus to date. It provides new insights into the diversity and evolution of organellar genomes of an iconic, speciose clade of lichenized fungi (i.e., the reindeer lichens, *Cladonia* subgenus *Cladina*), as well as more broadly a keystone group of ecologically important, terrestrial obligate symbionts (i.e., lichens). Our observations of the relationship between fungal life strategy and mitochondrial genome size highlight how genomic streamlining via the coevolution of an obligate symbiosis does not correlate to a reduction in mitochondrial genome size. The rare emergence of ATP9 duplications documented here in *Cladonia ravenelii* and *Stereocaulon pileatum* are also interesting phenomena that have not been seen in other lichens. Finally, this study also shed light onto the considerable variation in HEG presence and abundance in *Cladonia* and the stark contrast of the comparatively HEG-poor genus *Lepraria*. These observations draw further questions on the nature of the symbiosis of these selfish mobile sequences and their host genes. Ultimately, this study provides a framework for comparative studies of organellar genomes in other lichen lineages and advances the

integration of lichens into broader studies of organismal genomics and evolution.

ACKNOWLEDGMENTS

This research was conducted as part of the doctoral dissertation of J.R.H. Christopher Blair, Elizabeth Alter, Jessica Allen, and Yoshihito Ohmura were influential advisors on this research and as such deserve a great thanks. Some collections and sequence data were provided by the Kane and Tripp laboratories at the University of Colorado Boulder. Collections from Japan were acquired with the assistance of Evgeny Davydov, Irina Galanina, and Alexander Paukov, as well as the Ohmura laboratory at the Tokyo Museum of Nature and Science. Additional thanks are extended to Troy McMullin, John Franklin, Jim Battaglia, and Carly Anderson for their assistance in collections and sequence data generation. Finally, we thank Jennifer Dorey, Robin Sleith, Carlos Rodrigues-Vaz, Simon Verlynde, Lydia Paradiso, Charlie and Katie Zimmerman, and Sarah Hardy for supporting the authors throughout this project.

DATA ACCESSIBILITY STATEMENT

Raw sequence reads are deposited in the SRA (BioProjects PRJNA731936 and PRJNA770763). Individual assembled mitochondrial genomes are deposited in GenBank under accession numbers OL989692–OL989772 (TABLE 1).

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

FUNDING

This work was supported by the National Science Foundation [#1432629]; National Science Foundation [1542639]. This research was funded by multiple sources, including the National Science Foundation (NSF) dimensions project grants 1432629 (to James Lendemer) and 1542639 (to collaborators at the University of Colorado, Boulder) and the Japan Society for the Promotion of Science (JSPS) short-term fellowship.

ORCID

Jordan R. Hoffman http://orcid.org/0000-0001-6055-0678

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